The Cystine-Rich Envelope Protein From Human Epidermal Stratum Corneum Cells

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A cystine-rich protein has been extracted from the membrane region of stratum corneum cells with 50 mM Tris-HCl buffer, pH 7.3. The protein has been purified by molecular sieve chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and preparative isoelectric focusing. An antibody, raised to the purified protein, was located in the cell membrane region in the stratum spinosum, stratum granulosum, and the inner part of the stratum corneum. In addition, the antibody also reacted with the protein extracted from the outer part of the stratum corneum by the immunoblotting method. Amino acid analysis of the protein revealed a distribution of 4.3% cystine residues, 9% lysine, 18.5% glycine, and 12.6% glutamic acid residues. The isoelectric point was found to be 4.8 and the molecular weight of the protein was 16,000.
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The cell membrane proteins of epidermal cells show dramatic changes during keratinization. During the final stage of keratinization, ϵ-(γ-glutamyl-lysine) bonds [1–8] and disulfide bonds [9–12] are formed on the membranes of the stratum corneum cells and electron-dense bands are formed internal to the stratum corneum cell membranes [13].

Staining of human epidermis with a thiol-specific, fluorescent reagent, 7-(N-dimethylamino-4-methyl-3-coumarinyl) maleimide (DACM), revealed strong fluorescence attributed to disulfide bonds of polypeptides on the stratum granulosum and stratum corneum cell membrane region [9–11].

In previous reports, the $\frac{1}{2}$-cystine content of 8 M alkaline urea-insoluble fractions of the stratum corneum were calculated to be approximately 5% or 10% by Matoltsy and Matoltsy [13] and Hirotani et al [9], respectively. However, when the disulfide bonds of proteins in the solubilized fractions of the stratum corneum with the various extracting solutions (Tris-HCl buffer, pH 7.3, 4 M urea, pH 7.3, and 8 M urea, pH 9.0 solutions) were reacted with DACM and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), strong fluorescent protein bands were seen in only the 50 mM Tris-HCl buffer, pH 7.3, extractable fraction [12]. Thus, we chose to extract, purify, characterize, and localize the cystine-rich proteins (CRP) in the stratum corneum.

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Abbreviations:
CRP: cystine-rich protein(s)
DACM: 7-(N-dimethylamino-4-methyl-3-coumarinyl) maleimide
ELISA: enzyme-linked immunosorbent assay
FISA: fluorescent immunosorbent assay
FITC: fluorescein isothiocyanate
HRP: horseradish peroxidase
PMSF: phenylmethylsulfonyl fluoride
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TS: supernatant fraction

MATERIALS AND METHODS

Materials Normal stratum corneum was obtained from the sole skin of the feet. Sepharose 6B and an isoelectric focusing kit were obtained from Pharmacia Fine Chemical, Inc., of Fairlawn, New Jersey. Ultrodex and ampholines were provided by the LKB Company of Sweden. Goat antirabbit IgG serum (IgG fraction) which was labeled with fluorescein isothiocyanate, and fluorescein conjugate IgG fraction of goat anti-horseradish peroxidase (HRP) were purchased from Cappel Laboratory, Cochranville, Pennsylvania. All other chemicals were reagent or analytical grade and obtained from various commercial sources.

Preparation and Extraction of Tissues Immediately after being removed by a scalpel blade, the sheet of the stratum corneum was frozen in liquid nitrogen, lyophilized, and powdered in a Thomas-Wiley mill. The powder was stored at −20°C until needed.

For determining the best extracting solution, the CRP were extracted in steps with 3 solutions: (1) 50 mM Tris-HCl buffer, pH 7.3, containing 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Sup-1); (2) 50 mM Tris-HCl buffer, pH 7.3, containing 4 M urea and 10 μg/ml PMSF (Sup-2); and (3) 50 mM Tris-HCl buffer, pH 9.0, containing 8 M urea and 10 μg/ml PMSF (Sup-3). Extractions were at room temperature for 30 min followed by centrifugation at 15,000 g for 10 min (Tominaga refrigerated automatic centrifuge). The resultant supernatant fraction was analyzed in SDS-PAGE after sulfhydryl groups were blocked with N-ethyl-maleimide and disulfide bonds were reduced and conjugated with DACM reagent.

In order to check the extraction histochemically, we embedded both nonextracted powder and the pellet, which had been extracted for 3 days in 50 mM Tris-HCl buffer and centrifuged, in a Tissue-Tek OCT compound 4583 and prepared 6-μm sections. Sulfhydryl groups were blocked and disulfide bonds were reacted with DACM as above, and the fluorescence was examined.

In order to collect CRP, the lyophilized powder of the stratum corneum was extracted in 50 mM Tris HCl-buffer, pH 7.3, containing 10 μg/ml PMSF for 15 h at room temperature, followed by centrifugation at 15,000 g for 10 min. The resultant supernatant fraction (TS fraction) was further fractionated. In order to obtain

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CRP from the stratum granulosum, the outer, middle, and inner stratum corneum and the normal plantar skin of an amputated leg were minced into 5-mm^2 pieces and the pieces embedded into OCT compound. Sixteen micro-thick, horizontal sections (parallel to the surface) were prepared, dried, and divided into the 4 fractions: the outer, the middle, and the inner part of the stratum corneum and the stratum granulosum-rich fractions as determined by light microscopy. The sections were stored at −20°C until needed.

Gel Filtration Studies The TS fraction was subjected to gel filtration on a column of Sepharose 6B (0.9 × 50 cm). The eluant was 50 mM Tris-HCl at pH 7.9 containing 6 M guanidine hydrochloride and 10 mM 2-mercaptoethanol. The elution profile was monitored at both 280 and 480 nm.

The TS fraction, which had previously been conjugated with DACM, was subjected to Sepharose 6B column chromatography to determine the number of the fraction in which the CRP was eluted. Fluorescence was measured by fluorescent spectroscopy (Hitachi MPF-4). The excitation wavelength was 400 nm and emission at a wavelength of 480 nm was recorded.

Sodium Dodecyl Sulfate-Polyacrylamide Slab Gel Electrophoresis and Isoelectric Focusing The SDS-PAGE was carried out in 12.5% gel by the method of Laemmli [14] in the absence of urea. After the SDS-PAGE gel was stained with Coomassie Brilliant Blue R-250, the specific protein bands from SDS-PAGE gel were excised. Protein collection was accomplished through electrophoresis. After SDS was removed by the technique of Henderson et al [15], the material was subjected to isoelectric focusing using Ampholine pH 3.5–10.

Amino Acid Analyses Amino acid analyses were conducted following hydrolysis at 110°C, in vacuo, for various periods of time in the presence of 6.0 M redistilled HCl. Half-cystine concentration was determined by S-sulfo cystine using Inglis and Liu's method [16].

Immunologic Procedures The protein fraction obtained through SDS-PAGE and isoelectric focusing was at first used to immunize rabbits. No production of antibody was obtained after having repeated injections for more than 6 months, either because of its low molecular weight or its low antigenicity. Therefore, the purified protein was conjugated with HRP according to Wilson and Nakane's method to form a high-molecular-weight protein [17]. After the crude protein–HRP complex was purified by Sephadex G-200 column chromatography, it was emulsified with Freund's complete adjuvant and injected into rabbits (1 mg of protein in a 1.0 ml emulsion per animal). The animals were bled and boosted at appropriate intervals.

Sera were assayed for precipitating antibody using the Ouchterlony double-diffusion technique and the fluorescent immunosorbent assay (FISA) utilizing fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG immunoglobulin (IgG fraction).

Fluorescent Immunosorbent Assay We used FISA to determine the formation of antibody against the CRP, because false-positive reactions were observed when HRP-labeled antirabbit goat antiserum was used. The FISA was modified by the method of ELISA (enzyme-linked immunosorbent assay) according to the technique of Voller et al [18]; for the detection of the antigen–antibody reaction, the FITC-labeled goat antirabbit IgG globulin (IgG fraction) was used instead of utilizing HRP-labeled goat antirabbit IgG globulin.

Indirect Immunofluorescent Studies Indirect immunofluorescence studies were conducted according to Kimura's technique [19] using normal plantar and forearm skin. These were frozen and cut in a cryostat and fixed in cold 98% ethanol for 3 min. A 1:120 dilution of antibody with FITC-labeled goat antirabbit IgG globulin and the FITC-labeled anti-HRP goat IgG globulin were used.

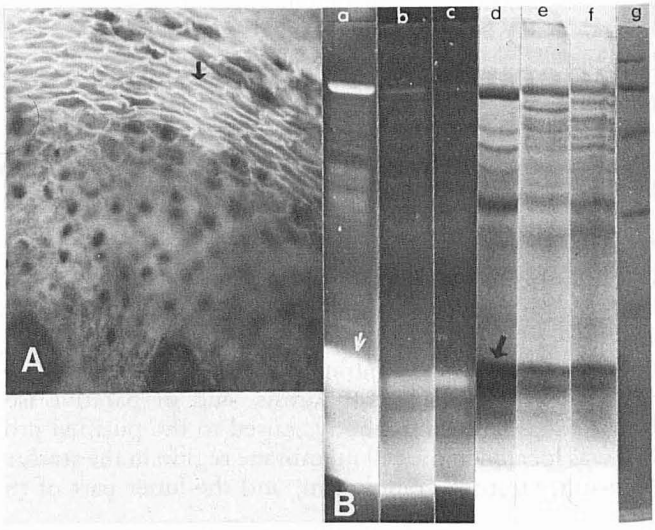

Figure 1. Fluorescence due to the disulfide bonds with the thiol-specific fluorescent reagent, DACM, and SDS-PAGE. *A*, Reticular fluorescence in the stratum corneum due to disulfide bonds conjugated with DACM (*arrow*). *B*, Fluorescence patterns of the 15,000 *g* supernanant fractions [Sup-1 (*a*), Sup-2 (*b*), and Sup-3 (*c*)] in SDS-PAGE. An equal volume of 3 supernatant fractions were combined with DACM and subjected to SDS-PAGE. Two protein bands which were stronger in intensity of fluorescence (*arrow*) were present in Sup-1 (*A*). Both were extractable in Tris-HCl buffer at pH 7.3. *d–f*, SDS-PAGE of the 15,000 *g* supernatant fraction [Sup-1 (d), Sup-2 (*e*), Sup-3 (*f*)]. Coomassie Brilliant Blue R stain. *g*, Molecular weight markers: phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and alpha-lactalbumin (14.4K), from the top.

Immunoblot Studies After the Tris-HCl buffer extracts from the outer, middle, and inner parts of the stratum corneum and the stratum granulosum-rich fraction were subjected to SDS-PAGE analysis, immunoblotting was done in accordance with the method of Gershoni et al [20–22].

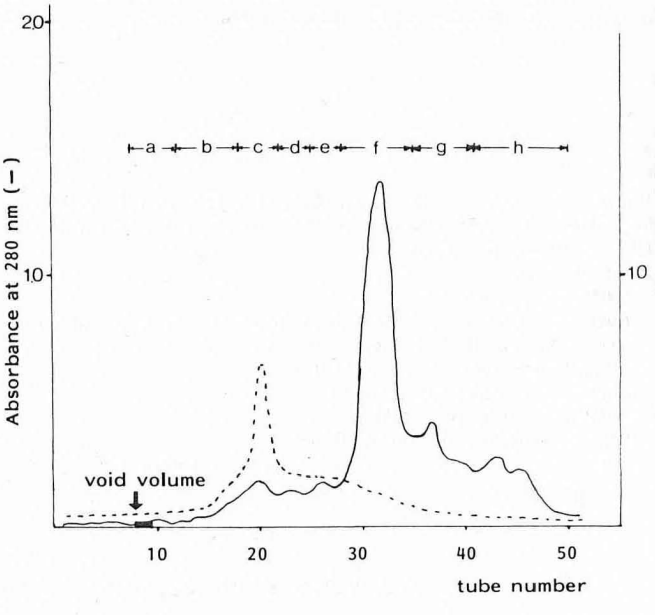

Figure 2. Molecular sieve chromatography (Sepharose 6B) from the 15,000 *g* supernatant fraction of Tris-HCl extract (TS fraction). When this supernatant, which was previously conjugated with DACM, was eluted, the fluorescent fraction eluted in the c-fraction in tubes 18–22.

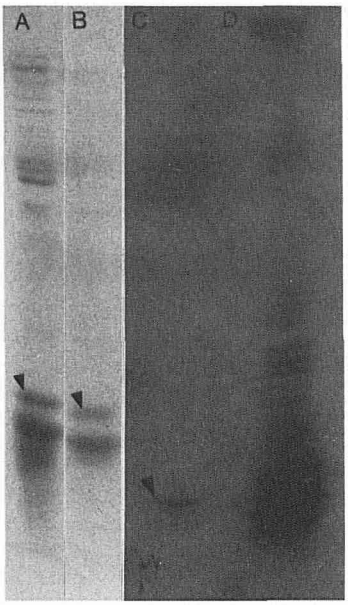

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic profiles and isoelectric focusing patterns. *A*, SDS-PAGE profile of the TS fraction. *B*, SDS-PAGE profile of the c-fraction from Sepharose 6B column chromatography (*c* in Fig 2). *C*, The protein band in (*B*) was excised from a gel and subjected to the isoelectric focusing after SDS removal. *D*, Isoelectric focusing profile of the TS fraction. *Arrowheads* indicate the cystine-rich, fluorescent protein band.

RESULTS

Extraction of CRP from the Stratum Corneum As the fluorescent materials at the cell membrane region (which was due to disulfide bonds reacted with DACM) were almost removed after 3 days' extraction in the 50 mM Tris-HCl buffer, pH 7.3, and in addition to this, the fluorescent protein bands were detected in SDS-PAGE of the 15,000 *g* supernatant of the 50 mM Tris-HCl solution after 30-min extraction (Fig 1), the CRP was extracted mostly by 50 mM Tris-HCl buffer, pH 7.3.

The TS fraction was then subjected to column chromatography using Sepharose 6B. The fluorescent peak (due to previous conjugation of the TS fraction with DACM) eluted in a fraction c (Fig 2) following the void volume. It was collected and lyophilized.

Table I. Amino Acid Analyses

	20 Hours	70 Hours
Half-cys	4.30	4.27
Asp	8.25	8.28
Thr	5.35	5.36
Ser	9.77	9.73
Glu	12.68	12.72
Pro	Trace	Trace
Gly	18.54	18.04
Ala	5.33	5.35
Val	5.25	5.18
Met	Trace	Trace
Ile	3.66	3.67
Leu	6.78	6.76
Tyr	Trace	Trace
Phe	2.91	2.90
Try	ND	ND
Lys	9.04	9.18
His	2.25	2.25
Arg	6.32	6.29

The purified protein was subjected to amino acid analysis using the technique described in the text. Results are expressed as pecentages of total residues. Hydrolyses were performed for 20 and 70 h. Cysteic acid content was determined as S-sulfocysteine; ND = not determined.

Electrophoretic Studies In order to determine the best extracting solution for CRP, the disulfide bonds of the 3 supernatant fractions (Sup-1, Sup-2, and Sup-3) were reduced and reacted with DACM. Aliquots of the 3 extracts were subjected to SDS-PAGE [Sup-1 (a,d), Sup-2 (b,e), and Sup-3 (c,f) in Fig 1*B*]. Two protein bands were remarkably strong in fluorescence, both of which were extracted with Tris-HCl buffer at pH 7.3. The lower-molecular-weight protein band (arrowhead) was detected in SDS-PAGE of the fraction c which was eluted from Sepharose 6B column (Fig 3*B*). This Coomassie Brilliant Blue R-stained protein band was excised from a SDS-PAGE gel and the protein was electrophoretically collected and subjected to isoelectric focusing

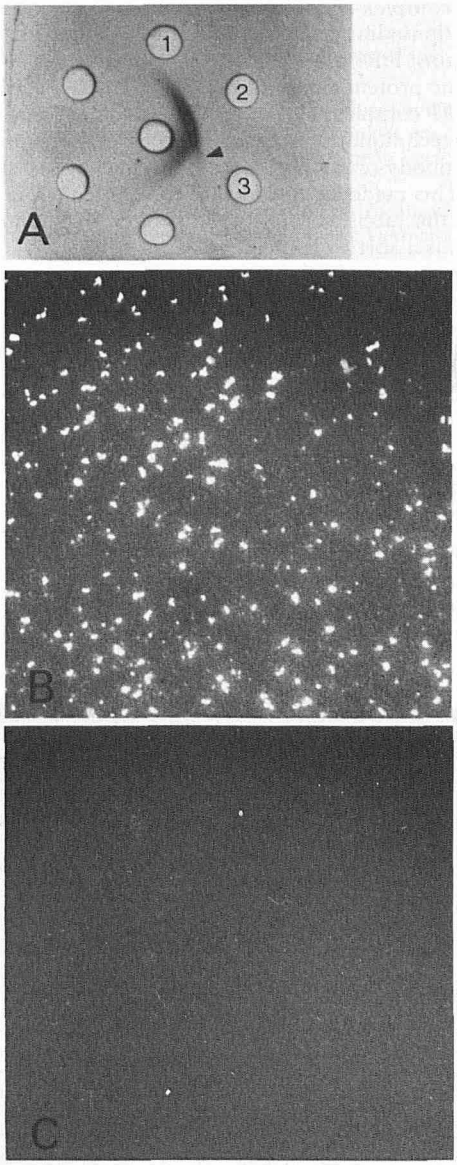

Figure 4. Detection of antibody against the purified CRP. *A*, Analysis by double diffusion in agar of antibodies produced against the CRP-HRP conjugate. The *center well* contains rabbit antiserum raised to CRP-HRP conjugate. The peripheral wells contain CRP alone (*1*), CRP-HRP conjugate (*2*), and HRP alone (*3*). Specific antibody was formed against the CRP-HRP conjugate. *Arrowhead* indicates spur formation. *B and C*, Analyses by FISA. *B*, The antigenic protein, antiserum (rabbit), and FITC-labeled goat antirabbit IgG globulin (IgG fraction) were reacted in steps. *C*, Preimmunized rabbit serum was used instead of antiserum as a control. Positive reaction was observed in (*B*) under fluorescence microscopy.

after SDS removal; only a single protein band with an isoelectric point at 4.8 was found (Fig 3*C*). The fraction at pH 4.8 was used for amino acid analyses and antibody production.

Results of Amino Acid Analyses Results from amino acid analyses performed upon purified CRP material were tabulated (Table I). Hydrolyses were continuous over 20- and 70-h periods. The most common amino acids were glycine (18.5%) and glutamic acid (12.7%). The proportion of basic amino acid residues was 17.5%: histidine, 2.2%; arginine, 6.3%; and lysine, 9.0%. The content of half-cystine was 4.3%.

Immunologic Studies To examine the in vivo location of the purified protein, immunologic studies were carried out. Results indicated the protein–HRP complex was antigenic in rabbits, eliciting precipitating antibodies with the formation of a single line of precipitation in agar when assayed with the antigenic protein–HRP complex (Fig 4*A*). However, as this antigen–HRP antibody precipitin line was partially identical to the HRP line when assayed against HRP alone, and no precipitin line occurred against the antigenic protein alone, the antibody reacted chiefly with the antigen–HRP complex. However, when the more sensitive method (the FISA technique) was used, we were able to prove that an antigen–antibody reaction occurred with the purified protein alone (Fig 4*B*). This evidence means that the antihapten antibody was formed in the rabbit serum as the antigen in the antigen–HRP complex was a sort of hapten.

The in vivo origin of the extracted material was ascertained definitively by immunofluorescent studies using an indirect technique (Fig 5). Specific fluorescence was apparent at the periphery of cells from the stratum spinosum, stratum granulosum, and inner part of the stratum corneum for both forearm and plantar skin (Fig 5*A,C*). Fluorescence was eliminated through the absorption of antiserum by purified material, though not by HRP alone (Fig 5*D*). Neither preimmune, normal rabbit serum (Fig 5*B*) nor fluorescein-conjugated IgG fraction of goat anti-HRP serum showed any specific fluorescence.

Immunoblotting Results A single protein band was detectable when the purified, 16K protein was applied to SDS-PAGE and stained with Coomassie Brilliant Blue R (Fig 6*F*). However, 2 protein bands reacted with the antibody serum in immunoblotting analysis (Fig 6*E*). The 16K CRP, reactable with the antibody, was found in all extracts from the 3 layers of the stratum corneum (outer, middle and inner parts) and from the stratum granulosum (Fig 6*A–D*). Besides the 16K protein, 4 other positive protein bands (64K, 57K, 48K, and 42K) were seen in the extracts from the middle and inner part of the stratum corneum, but not in the outer layer extract (Fig 6*A*). However, the 16K protein and a single high-molecular-weight protein band were positively seen in the granular layer extract (Fig 6*D*).

DISCUSSION

We feel certain the cystine-rich, buffer-soluble material, which was located at the cell membrane region, was almost entirely removed during our extraction procedures, because the fluorescence due to disulfide bonds of polypeptides, which was located at the cell membrane region of the stratum corneum in the tissue specimen, almost completely disappeared after 3 days' extraction. Purified CRP was, indeed, derived from one of the buffer-soluble CRP from the stratum corneum as the purified CRP was proved to be immunologically identical to the low-molecular-weight cystine-rich, fluorescent protein band by the immunoblotting technique. The antibody produced from the purified CRP, however, appeared to localize in only one part of the same region as the DACM-fluorescent material of the membrane region using an indirect immunofluorescent technique. Why only a part and not the whole stratum corneum showed fluorescence under the immunofluorescence technique remains unknown.

However, as the protein reactive with the antibody and having the same molecular weight was found in an extract from the outer part of the stratum corneum via the immunoblotting technique, it may be that the antigenic site was blocked in vivo in the outer part of the stratum corneum. Perhaps because of this blockage, no fluorescence was observed when human skin was examined with the indirect immunofluorescent technique.

Although there was a high-molecular-weight protein band evident (64K) when the purified CRP was subjected to SDS-PAGE and analyzed by the immunoblotting technique, it may be the tetramer of the purified 16K protein as its molecular weight was 64K (64 = 16 × 4), and the purified CRP was excised from a 16K protein band in a SDS-PAGE gel. However its amount must

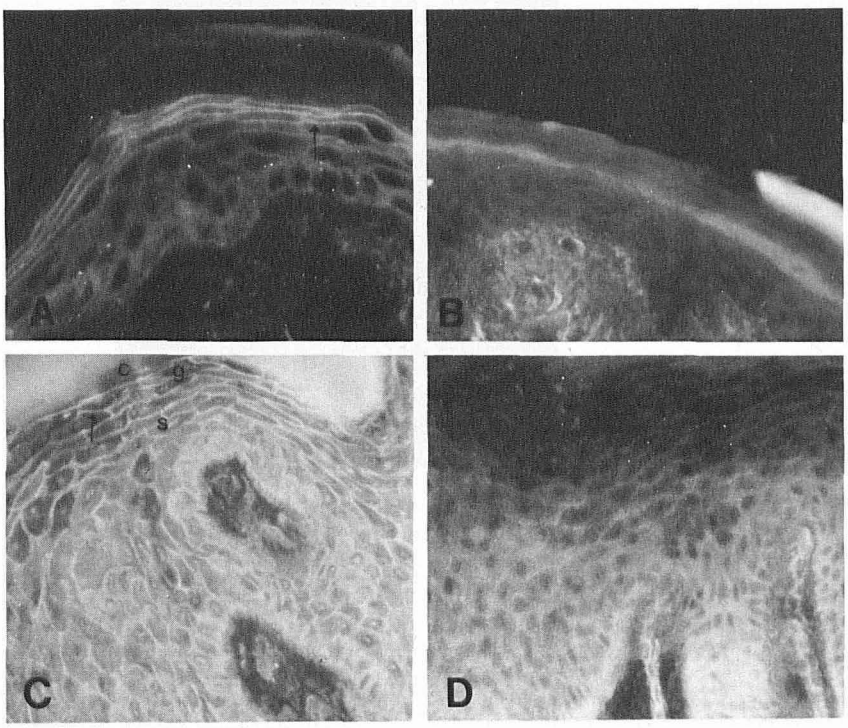

Figure 5. Analyses by indirect immunofluorescence of the antibody produced to the CRP. Human forearm (*A*) and sole (*C*) skin were fixed in ethanol, incubated with anti-CRP antibody, and stained with FITC-labeled goat antirabbit antiserum (1:120 dilution). *A*, Specific fluorescence (*arrow*) was observed at the periphery of cells in the stratum spinosum, stratum granulosum, and inner part of the stratum corneum (forearm skin). × 230. *B*, Tissue (forearm skin) incubated with control, preimmune rabbit serum. × 120. *C*, Specific fluorescence (*arrow*) was seen at the periphery of the stratum spinosum (*s*), stratum granulosum (*g*), and stratum corneum (*c*) (plantar skin). × 230. *D*, Tissue (plantar skin) incubated with the antiserum that had been previously absorbed with HRP. Fluorescence was still observed on the membrane region. × 230.

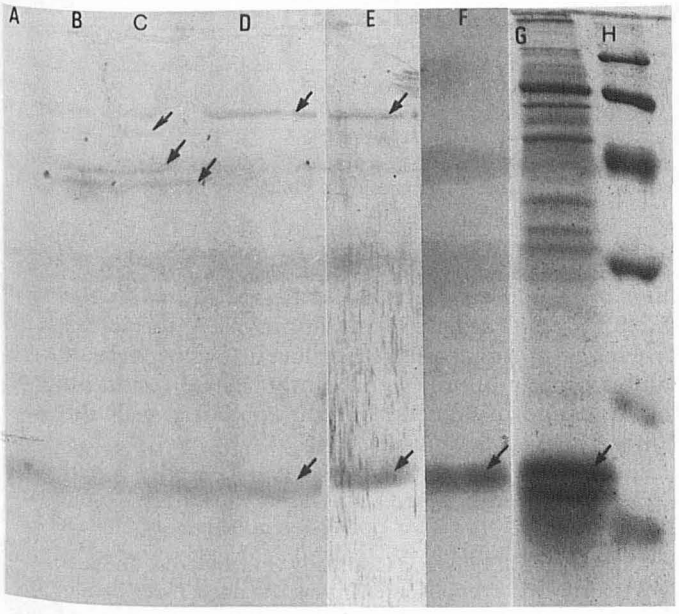

Figure 6. Analysis of the reaction of antiserum with the extract from the stratum corneum by the immunoblotting technique (*A–E*) and Coomassie Brilliant Blue R stain (*F–H*). Extract from the outer (*A*), the middle (*B*), and the inner (*C*) part of the stratum corneum and from the stratum granulosum (*D*). *E*, The purified CRP was subjected to SDS-PAGE and analyzed by immunoblotting technique. *F*, The purified CRP was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R. *G*, The 15,000 *g* supernatant fraction of the TS fraction was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R stain. *H*, The 16K protein band reactable with the antibody was observed in the whole cell layer of the stratum corneum. Besides this protein band, several high-molecular-weight protein bands reacted with the antibody (*arrow*). Molecular weight markers: phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and alpha-lactalbumin (14.4K), from the top.

be very small because when the purified protein was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R stain, no such high molecular protein was seen. On the other hand, when the extracts from the 3 parts of the stratum corneum and the stratum granulosum were subjected to SDS-PAGE and analyzed by the immunoblotting technique, other high-molecular-weight proteins were detected besides the extract from the outermost cell layers. Although these were immunologically common with the purified CRP, these protein bands could not be explained as the polymers of the purified protein. The nature of these high-molecular-weight proteins and the reason why these exist only in the middle and inner parts of cell layers and not in the outer part of cell layer remains unknown and could be solved in future studies. The possibility that the 57K to 42K proteins may be the degradation products of the 64K protein is suggested.

Though this protein is glycine-, glutamic acid-, and aspartic acid-rich in amino acid composition, its composition differs from that of membrane proteins from epidermal cells reported by Lobitz et al [7] and Rice and Green [5]. Those proteins contained less than 1% cystine.

In contrast to the preparative technique outlined in this paper, the cystine-rich components reported by Matoltsy and Matoltsy [13] and Hirotani et al [9] were residue fractions obtained after 1 M NaOH or 8 M alkaline urea extractions. Both residues manifested marginal bands when viewed under electron microscopy. In our investigation the marginal bands remained intact throughout the extraction process and, therefore, our CRP differed from that of the aforementioned authors.

The CRP presented in this report is a unique and new protein which may play an important role in the biologic functions of the stratum corneum.

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